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BARD Project Number: US -2759-96

Project Title: Use of anti-fungal gene synergisms for improved foliar and fruit disease tolerance in transgenic grapes

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Disease resistance, Vitis, grapevine, lytic peptide, chitinase, genetic engineering

Abbreviations commonly used in the report, in alphabetical order**:**

Budget: IS: $139300 US: $160700 Total: $300000

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		1	4	5
Submitted, in review, in preparation		2		2
Invited review papers				
Book chapters		1	1	2
Books				
Master theses				
Ph.D. theses		1		1
Abstracts		3	3	6
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings			1	1
Longer Visits (Sabbaticals)	1			1

Description of Cooperation:

Frequent email communication.

Sharing of results and techniques as soon as possible.

Coordination of efforts during sabbatical leave in Israel of the US partner, and during 1998 meeting in France.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

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Original objectives

1. Test anti-fungal gene products for activity against *Uncinula necator*, *Aspergillus niger*, *Rhizopus stolonifer* and *Botrytis cinerea*.
2. For *Agrobacterium* transformation, design appropriate vectors with gene combinations.
3. Use biolistic bombardment and *Agrobacterium* for transformation of important cultivars.
4. Characterize gene expression in transformants, as well as level of powdery mildew and *Botrytis* resistance in foliage of transformed plants.

Background

The production of new grape cultivars by conventional breeding is a complex and time-consuming process. Transfering individual traits via single genes into elite cultivars was proposed as a viable strategy, especially for vegetatively propagated crops such as grapevines. The availability of effective genetic transformation procedures, the existence of genes able to reduce pathogen stress, and improved in vitro culture methods for grapes, were combined to serve the objective of this proposal. Effective deployment of resistance genes would reduce production costs and increase crop quality, and several such genes and combinations were used in this project.

Progress

The efficacy of two-way combinations of *Trichoderma* endochitinase (CHIT42), synthetic peptide ESF12 and resveratrol upon the control of growth of *Botrytis cinerea* and *Penicillium digitatum* were evaluated in vitro. All pairwise interactions were additive but not synergistic. Per objective 2, suitable vectors with important gene combinations for *Agrobacterium* transformation were designed. In addition, multiple gene co-transformation by particle bombardment was also tested successfully. In New York, transformation work focused on cultivars Chardonnay and Merlot, while the technology in Israel was extended to 41B, R. 110, Prime, Italia, Gamay, Chardonnay and Velika. Transgenic plant production is summarized in the appendix.

Among plants developed in Israel, endochitinase expression was assayed via the MuchT assay using material just 1-5 days after co-cultivation. Plants of cv. Sugraone carrying the gene coding for ESF12, a short anti-fungal lytic peptide under the control of the double 35S promoter, were produced. Leaf extracts of two plants showed inhibition zones that developed within 48 h indicating the inhibitory effect of the leaf extracts on the six species of bacteria. *X. fastidiosa*, the causal organism of Pierce's disease, was very sensitive to leaf extracts from ESF12 transformed plants. Further work is needed to verify the agricultural utility of ESF12 transformants. In New York, some transformants were resistant to powdery mildew and *Botrytis* fruit rot.

Major conclusions, solutions, achievements and implications

The following scientific achievements resulted from this cooperative BARD project:

1. Development and improvement of embryogenesis and tissue culture manipulation in grape, while extending these procedures to several agriculturally important cultivars both at Israel and USA.
2. Development and improvement of novel transformation procedures while developing transformation techniques for grape and other recalcitrant species.

3. Production of transgenic grapevines, characterization of transformed vines while studying the expression patterns of a marker gene under the control of different promoter as the 35S CaMV in different part of the plants including flowers and fruits.
4. Expression of anti-fungal genes in grape: establishment of transgenic plants and evaluation of gene expression. Development of techniques to insert multiple genes.
5. Isolation of novel grape specific promoter to control the expression of future antimicrobial genes.

It is of great importance to report that significant progress was made in not only the development of transgenic grapevines, but also in the evaluation of their potential for increased resistance to disease as compared with the non engineered cultivar. In several cases, increased disease resistance was observed. More research and development is still needed before a product can be commercialized, yet our project lays a framework for further investigations.

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ACHIEVEMENTS

World production of grapes is in excess of 65 million metric tons, exceeding the production of all other temperate fruits, and surpassed only by *Citrus* and banana among all other fruit crops around the world. Grapes are grown on over 9 million hectares. While the major portion is consumed as wine and spirits, grapes are also used in quantity as fresh fruit and dried as raisins. The importance of powdery mildew resistance in grapevines cannot be underestimated. The disease causes the greatest amounts of damage among all fungal diseases worldwide. Where more organic measures such as sulfur are used for control, weekly applications are often necessary, and the level of control is not sufficient. Frequently applied pesticides also require a large expenditure in time, labor, and fuel costs. Resistance would reduce production costs and increase crop quality. We achieved results that should be translated into agricultural progress through the availability of resistant varieties. Techniques have also been developed that will result in further progress in understanding grapevine genetics. Transformation techniques can be used to study gene function and many grapevine genes are being cloned in labs around the world. A greater understanding of their function will be of immense benefit in both basic and applied studies.

ESTIMATED IMPACT

Production costs for grapes in Israel are between 8,000-10,000 US$ per hectare. About 30% of these costs are due to the need for fungal protection performed mainly by spraying. In case the transformed grape plants would exhibit a full protection against fungal diseases; a significant reduction in production costs (up to 30%) might be expected. If only a limited protection will be obtained using the transgenic grapes produced in this program, one might expect a 10-15% reduction in production costs. In the US, production costs for disease control are lower. However, even a 5% reduction in costs on an annual basis would help maintain the competitive position of growers in an increasingly global economy.

As a result of improvements on co-transformation procedures, cryopreservation techniques, promoter cloning, and other tissue culture technology improvements, this project will impact favorably on many future works in grapevine improvement via

transformation. Functional genetic studies will also be further facilitated by the progress made during the course of this project.

DETAILS OF COOPERATION

The proposed project was planned in order to provided valuable information on the possibilities for the development of transgenic grapevines, which control fungal pathogens via expression of heterologous genes. Our eventual goal was to improve resistance to powdery mildew and other diseases of important varieties with the insertion of relevant genes and gene combinations. At the end of this project, transgenic plants are undergoing testing. These should provide data on field resistance and stability of expression of the foreign genes. It is our ultimate goal to develop transgenic grapes that will be more efficient and economical for growers to produce, while also providing consumers with familiar products grown with reduced chemical inputs.

Since 1997, the PIs have been collaborating on this subject and share the grape cultivars to be transformed. Both PIs have kept an open email line for real-time discussions. Techniques have been shared between both groups, as have plasmid constructs and genes of interest (subject to restrictions from those controlling rights to these genes). In 1998, the PIs met at a conference in Montpellier, France, affording an opportunity to communicate during the early stage of this project. During February to July, 1999, B.I. Reisch spent a 6 month sabbatical leave in the lab of Dr. Perl at Volcani Center. We consulted with each other extensively during this period, and progress was made toward the development of a multi-gene vector for transformation.

List of Publications

Ali, G.S. 1998. Genetic transformation of grape (*Vitis* sp.) with the *Trichoderma* endochitinase gene, and interactions of endochitinase, a synthetic peptide and resveratrol in controlling fungal growth in vitro. Ph.D. Thesis, Cornell University, Ithaca, NY.

Ali, G.S., G.E. Harman, and B.I. Reisch. 2002. The interaction of endochitinase,a synthetic peptide and resveratrol in controlling fungi in vitro. (submitted to European Journal of Plant Pathology)

Colova-Tsolova, V., A. Perl, S. Krastanova, I. Tsvetkov, and A. Atanassov. 2001. Genetically engineered grape for disease and stress tolerance. In: Roubelakis-

Angelakis, K. (ed.). Molecular Biology and Biotechnology of Grapevine. Kluwer Academic Press, Netherlands. pp. 411-432

Gollop, R., S. Farhi, and A. Perl. 2001. Regulation of the leucoanthocyanidin dioxygenase gene expression in *Vitis vinifera*. Plant Science 161:579-588.

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Kikkert, J.R., M.R. Thomas and B.I. Reisch. 2001. Grapevine genetic engineering. In: Molecular Biology and Biotechnology of Grapevine, Kalliopi A. Roubelakis-Angelakis (Ed.), Kluwer Academic Publishers, the Netherlands, pp. 393-410.

Perl, A., N. Sahar, P. Spiegel-Roy, S. Gavish, R. Elyasi, E. Or and H. Bazak. 2000. Conventional and biotechnological approaches in breeding seedless table grapes. Proceedings of the VIIth International Symposium on Grapevine Breeding and Genetics, Montpellier, France, July 6-10, 1998, Acta Horticulturae 528:607-612.

Vidal, J.R., J.R. Kikkert, P.G. Wallace and B.I. Reisch. 2002. Biolistic co-transformation and regeneration of ‘Chardonnay’ (*Vitis vinifera* L.) with antimicrobial peptide genes. (submitted to Transgenic Research).

Wang, Q., R. Gafny, N. Sahar, Ilan Sela, Munir Mawassi, Edna Tanne, Avihai Perl. 2002. Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. Plant Science 162:551-558.

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APPENDIX

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Unpublished data from Volcani Center

Design of vectors for constitutive expression of endochitinase, glucanase, resveratrol, ESF12 and RIP.

As planned during the project, several vectors were constructed. Most of them were very complex for preparation since they contain several antifungal genes on the same binary vector in order to achieve the expected synergism to combat pathogenesis.

The following vectors were produced within the frame of the project:

35S-CaMV- Endochitinase; 35S-CaMV- Glucanase; 35S-CaMV – RIP.
35S-CaMV – Antimicrobial ESF12; 35S-CaMV- Glucanase; 35S-CaMV – RIP.
PR-Promoter - Stilbene synthase
35S-CaMV - Antimicrobial ESF12
PR-10- Promoter - Stylbene synthase; -35S-CaMV - Glucanase; 35S-CaMV – RIP
35S-CaMV - Antimicrobial ESF12; - 35S-CaMV- Endochitinase.
35S-CaMV - Antimicrobial ESF12; - 35S-CaMV- Endochitinase; -
PR-Promoter - Stilbene synthase
PR-10- Promoter - Stilbene synthase ; - 35S-CaMV Superoxide dismutase; -
Ubiqitin- Endochitinase
9. 35S-CaMV – El301

The new constructs were utilized: the bacterio-opsin (*bO*) gene and the synthetic pCAE synthetic gene.
The *bO* gene (designated as EL 301) is a bacterial gene known to confer enhanced resistance in transgenic higher plants to pathogenic attack. This gene was isolated from *Halobacterium halobium* and was shown to function as a light-driven proton pump that utilizes a different light spectrum from that used by the photosynthetic apparatus of higher plant. Transgenic tobacco plants expressing the bO gene were found to have a systemic resistance against viral and bacterial pathogens such as TMV, TNV and *Pseudomonas syringeae*. New indication suggested that this gene might confer tolerance to fungal pathogens as well.
The ESF12 gene is a synthetic variant of a naturally occurring antimicrobial gene. This synthetic peptide inhibited the germination of conidia from *Cryphonectria parasitica, Fusarium oxysporum* and *Septoria musiva* as well as the growth of the bacteria *Agrobacterium tumefaciens, Ervinia amylovora* and *Pseudomonas syringae.*

Extension of transformation technology to other cultivars of importance.
Suspension cultures in 250 ml Erlenmeyer flasks with 50 ml liquid medium were developed from embryogenic tissue cultures established from anthers of commercial cultivars of *Vitis vinifera* L. In the frame of the project we further established embryogenic cell suspensions of the following cultivars: Richter 110, Prime, Italia, Gamay, Chardonnay and Velika.
A novel protocol was developed utilizing *in vitro* shoot-tips as the explant for induction of embryogenic calli. As for today we were utilizing anthers from unopened

flowers as the optimal explant to induce embryogenesis. Using in-vitro tips as a starting material is highly advantages since embryogenic tissue may be induce all year round and is not restricted to field flowering grape plants.

It should be noted that our transformation system is highly efficient. We have recently developed a double transformation system. We developed a procedure for co-cultivating embryogenic plant tissue with two agrobacteria, harboring two different genes of interest, each agrobacterium carrying a different selectable marker. Selection was carried out using simultaneously both kanamycin and hygromycin in liquid shaking cultures.

Long term maintanance of embryogenic cell lines in bioreactors

Suspension cultures in 125 ml Erlenmeyer flasks with 20 ml liquid medium were developed from embryogenic tissue cultures established more than three years ago from anthers of four commercial cultivars of *Vitis vinifera* L. ["Superior seedless" (SR), cv. 49, cv. "Red Globe"(RG) and cv. 902]. The cultures were grown on two orbital shakers (90 and 130 rpm) at 25±1^{o}C in a diffuse 16 h light. The proliferation liquid medium consisted of MS salts and vitamins and was also supplemented with 3-4% sucrose, 1 ppm 2,4-D, 2 ppm NOA, 5 ppm IAA, 0.2 ppm BA, with or without 1 ppm ABA. For morphogenic induction, the GM+NOA medium was used. It contained MS salts and vitamins and was also supplemented with 18 g/l maltose, 4.6 g/l glycerol and 1 ppm NOA. Regeneration and subsequent plant germination was obtained on MS medium supplemented with 0.1 ppm NAA, 0.25% Gelrite and 3% sucrose, with or without 0.1 ppm TDZ. The conical bubble type bioreactors with working volume from 0.2 l to 0.7 l and an aeration rate 1-1.5 vvm were used.

All 17 of the cell suspension lines obtained retained a high embryogenic potential, but they differ from each other by the number of subcultures required before regeneration and germination could take place. Some lines demanded for regeneration an additional stage of preliminary cultivation on the induction medium. Long-term cultivation on proliferation medium resulted in blockage of embryogenesis at early stages of development after transfer onto the regeneration medium. Utilization of the GM+NOA medium for the sub cultivation of these suspension culture lines resulted in a shift of the block to later stages of development or in its cancellation. Table 1 summarizes the different chart-flow established in order to regenerate plantlets from the different lines. The possibility of growing the suspension cultures of cvs. 902 and RG in the conical bubble type bioreactors in batch regime, and with further four (902) or six (RG) cycles of draw-fill culture regime was demonstrated in course of half a year cultivation. High level and low fluctuation of embryogenic activity of these suspension cultures grown in the bioreactors confirm susceptibility of this system for genetic transformation.

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Protocol for regeneration of the different lines of suspension cultures from four commercial cultivars of *Vitis vinifera* [cv. "Superior seedless" (SR), cv. 49, cv. "Red Globe" (RG) and cv. 902].

Line designation	Type of suspension	Subcultures required on media		% of regeneration culture up to ...
		A	B	on media C
RG - 1	Fine cells	6-10	0	50
RG - 2	Globular	3-10	0	50
RG - 3 (bioreactor)	Globular	1-7	0	70
RG - 5	Embryoids	0	4-6	90
902 - 1	Fine cells & small aggregates	7-8	0	60
902 - 1.1 (bioreactor)	Fine cells & small aggregates	1-5	0	65
902 - 2	Fine cells	0	3-4	70
902 - 3	Embryoids	0	4-7	85
SRI - 1	Fine cells	2-3	0	50
SRII - 1	Fine cells	5-10	0	55
SRII - 4	Embryoids	0	3-6	85
SRIII - 1	Fine cells	2-3	0	40
SRIII - 2	Fine cells	0	1-2	0
49 - 1	Fine cells	3-5	0	75
49 - 3	Fine cells	0	1-2	0

A - proliferation liquid medium;
B - morphogenic induction liquid medium (Lebrun & Branchard, 1987);
C - regeneration solid medium.

Transform cultivars with anti-fungal genes constructed in Objective 1.
Embryogenic lines of different *Vitis vinifera* were used for *Agrobacterium* mediated transformation. Selection systems utilized the inhibitors hygromycin and kanamycin, as appropriate to the vectors constructed. The following table summarizes the different transformation experiments done and the state of each transformation study.

Grape cultivar	Construct used	Selection system	Current status
Superior	ESF12	Kanamycin	Mature plants
Superior	ESF12+Glu+RIP	Kanamycin	Mature plants
41B	Glu+RIP	Hygromycin	Mature plants
41B	Stilbene synthase	Kanamycin	Mature plants
Chardonnay	EL 301	Kanamycin	Mature plants
Superior	Stilbene synthase +Glu+RIP	{Kanamycin Hygromycin}	Mature plants

Red-Globe	ESF12+endo	Kanamycin	Mature plants
41B	ESF12+endo	Kanamycin	Mature plants

Test for increases in foliar powdery mildew and botrytis resistance in transgenic plants.
Transient expression assays:
There were four separate experiments using 'Superior' embryogenic cultures that had been grown either in solid media, in liquid suspensions, or in liquid bioreactors. Transient endochitinase assays were performed using the MuchT assay. Expression was assayed 1-5 days after *Agrobacterium* transformation under different co-cultivation conditions. It was interesting to note that the expression of the endochitinase gene as measured by the MuchT assay can actually serve as a novel reporter gene in a transformed tissue. Thus we studied the involvement of different co-cultivation conditions on the expression of the introduced endochitinase. Transformed embryogenic tissues were homogenized and assayed for endochitinase activity. The influence of the following conditions were studied: 1. The influence of the pH of the media during co-cultivation. 2. The effect of acetosyringone on the time and level of endochitinase expression. In a parallel study, several stable transformed plants were selected and evaluate for their endochitinase activity from leaves harvested from *in vitro* shoots. In order to obtain comparable results between different plants, all endochitinase activities were normalized to the average activity in leaves of the control which was given the value of 1.

ESF12 transformants inhibit growth of Xylella
The ESF12 gene is a synthetic anti-microbial lytic peptide active against a broad spectrum of fungal and bacterial diseases. Mature leaves from transgenic plants carrying this gene were sampled and homogenized. Bacterial medium was supplemented with extracts originated from transgenic leaves and bacterial growth inhibition was studied. We have transformed *Vitis vinifera* cv. Sugraone with the gene coding for ESF12 under the control of the double 35S promoter. Leaves from mature greenhouse plants were extracted. Samples were loaded into wells on top of plates containing actively growing bacterial cultures. Two transgenic plants designated #1 and #2 were sampled, along with a control plant (C).
Inhibition zones that developed within 48 h indicated the inhibitory effect of the leaf extracts on the bacteria. As a positive control, commercial Nisin (Sigma) was used (data not shown).
Strong zones of growth inhibition were obtained when leaf extracts were tested against cultures of the following bacteria: *E. coli V. cholera*, *P. aeruginase*, *M. luteus* , *E. amylovora* and *Xylella fastidiosa*. It is important to mention mainly the last two bacteria: *Erwinia amylovora* is the causal agent of fire blight, an important bacterial pathogen of apples and pears. Transgenic pear expressing the same gene, recently developed in Israel, showed similar tolerance against *Erwinia* (Flaishman and Perl, unpublished results). *Xylella fastidiosa,*one of the target organism of this study, was very sensitive to leaf extracts from ESF12 transformed plants. Inhibition zones were even larger compared to *Erwinia,* indicating that ESF12 and other lytic peptides might be very promising for the control of *Xylella fastidiosa* (Fig. 1).

Figure 1. Inhibition of *Xylella fastidiosa* by extracts of ESF-12 expressing grapevines, "1" and "2". The control non transformed vine extracts are on the right side of the plate. On the y-axis (right side) is the volume of plant extract added to the bacterial growth medium. Arrow points to zone of bacterial growth inhibition between 10 and 20 µl of extract from transformant 1.

Test for antifungal resistance in transgenic plants.

The transgenic plants were tested to determine the level of resistance to powdery mildew. *In vitro* grown plantlets were used since they usually lack a well developed cuticle, thereby making them excellent targets for *U. necator*. *In vitro* plantlets were inoculated with conidial suspensions. They were examined after 2 weeks and 4 weeks for percent leaf surface area infected, and density of mycelial growth. Necrotic reactions, indicating a possible hypersensitive response, were also noted. Where mycelial growth was observed, the time from inoculation to sporulation was recorded.

All *in vitro* tests were replicated at least 3 times on at least 4 plants of each transformant. Additional testing were performed on greenhouse acclimated potted vines, also replicated in the same fashion. Multiple isolates of *U. necator* were used in order to avoid the possible inaccuracy of results if a weak isolate were to be used in testing. In the greenhouse, mixtures of conidial sources were inoculated as 10 µl droplets and allowed to quickly dry in place. Colony size, necrosis, and mycelial density were noted after 2 and 4 weeks. Controls in all experiments included non transformed plantlets of the original cultivar. Several plants that expressed elevated levels of endochitinase were found to be also more tolerant to *U. necator* and results were correlated with level of expression. Additional tests in the following year, in the field, are required in order to determine if these plants are indeed more tolerant to *U. necator.*

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Additional studies performed within the frame of this research
During the course of the project, we faced several problems that were not anticipated when the project was submitted. We had to develop a novel method for the long term maintenance of embryogenic cells. These cells are the key factor for successful transformation and their optimal maintenance is highly crucial.
We developed a novel cryopreservation system enabling to freeze and thaw cell before transformation. It was surprising to see that cryopreserved cell are a better target for transformation and their regenerative potential is much higher. A paper describing the result of this part of the study is attached to this report.

Unpublished data from Cornell University

Testing of Antifungal Gene Products Against Grapevine Pathogens
In vitro testing of antifungal gene products showed that *Trichoderma* endochitinase, synthetic peptide ESF12, and resveratrol inhibited the growth of *Botrytis cinerea* and *Penicillium digitatum*. Combinations of these compounds also showed additive activity (Ali, Harman, and Reisch, submitted).

Design of Appropriate Vectors forTransformation.
Because the physical nature of the biolistic transformation process does not require specific DNA construction other than the basic promoter-gene-terminator sequences, we used vectors obtained from other labs (see list below).

Plasmid Designation	Promoters/Antimicrobial Genes	Marker
pBin19ESR	35S-35S-AMV-*Trichoderma* Endochitinase	nos-nptII
pBI121CNAG	35S-35S-AMV-*Trichoderma* Nagase	nos-nptII
pBin19CNAG+ENDO	35S-35S-AMV-*Trichoderma* Endochitinase 35S-35S-AMV-*Trichoderma* Nagase	nos-nptII
pCEAl	35S-ESF-12/Ac-AMP1.2	nos-nptII
pSAN167	Ubiquitin3-Magainin II	none
pSAN168	Ubiquitin3-MSI-99 synthetic peptide	none
pSAN315	Ubiquitin3-PGL	none
pSAN319	Ubiquitin3-MagII/PGL	none
pSAN237	none	ubq11-nptII

We also tested for the first time, the possibility of co-delivery of genes on different plasmids into grapevine cells. This would greatly reduce the time required for vector construction.

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Bruce Reisch spent a six-month sabbatical working with Avi Perl and Rachel Gollop at Volcani (February - July 1999). During this period, plans were made to develop a new vector with at least three antifungal genes, separate public domain (ubiquitin) promoters, and suitable for either *Agrobacterium* or biolistic transformation methods. The vector, when complete, will harbor the nagase, endochitinase, and magainin II genes, along with NPT II as a selectable marker. Intermediate stages in vector preparation were completed in Dr. Gollop's lab, and this work continues now at Cornell.

Development of Transgenic Grapevines using Biolistic Transformation
Embryogenic cultures of *Vitis vinifera* L. 'Chardonnay', 'Merlot', and 'Pinot noir' were transformed using the biolistic method previously developed in our laboratory for grapevine (Kikkert et al., 1996, Plant Cell Rep. 15:311-316). The following transgenic plants were obtained:

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	Plasmid	No.Transgenic Plants with DR gene(s)
Merlot:		
	pBin19ESR	74
	pBI121CNAG	1
	pBin19CNAG+ENDO	1
Chardonnay:	pBin19ESR	50
	PSAN167	16
	PSAN168	20
	pSAN315	21
	pSAN319	24
Pinot noir:	pSAN167, 168, 315, 319	0

Biolistic experiments that utilized pCEA1 did not yield transgenic plants due to technical difficulties with transformation and selection conditions used for those experiments.

Characterization of Transformants

a. Gene Expression and Molecular Analysis

Endochitinase Transformants:

Quantitative fluorescent chitinase assays were performed to measure the level of enzyme activity from leaves of in vitro-grown plants. Forty to fifty percent of the Merlot and Chardonnay lines expressed chitinase at levels 10 to 100 fold higher than non-bombarded controls. The presence of the ThEn42 gene in the chitinase positive lines was confirmed by PCR and Southern blot analysis. Nearly all of the plants tested contained fragments of the *ThEn42* gene. The plants that did not express chitinase either did not contain *ThEn42* at all or had only small fragments of the gene. Only one plant that contained the intact *ThEn42* gene did not express chitinase. All of the plants tested contained the *nptII* gene. Western blotting confirmed gene expression in both in vitro-grown and field-grown plants.

Magainin Transformants:

Chardonnay transgenic plants that contained magainin genes were produced by co-bombardment of the cells with gold particles coated with a 1:1 mixture of a) pSAN167 and pSAN237, b) pSAN168 and pSAN237, c) pSAN315 and pSAN237, or d) pSAN319 and pSAN237. Putatively transformed embryos were selected from kanamycin-containing medium. DNA was extracted from leaves of approximately 100 regenerated plants and was tested for the presence of the genes by PCR analysis.

The percent of *nptII* positive plants ranged from 70 to 84 percent, while the percent of co-integration ranged from 31 to 76 percent in different experiments. Dot blot analysis was used to further confirm the presence of the transgenes. The percent of npt-II positive lines ranged from 93 to 100 percent and the rate of magainin gene co-integration was between 48 to 63%.

b. Disease Resistance Screening of Endochitinase Transformants:

i. Botrytis leaf disc assays

A detached leaf assay was used to evaluate the response of transgenic grapevines to *B. cinerea*. Fully expanded leaves at position 3 from the apex of greenhouse-grown vines were placed, adaxial surface up, in styrene plastic boxes which were lined with moistened filter papers to maintain high humidity. Each leaf was inoculated with 5 µl droplets of a conidial suspension at a concentration of 10^5 conidia per ml. The conidial suspension consisted of a mixture of 4 isolates of *B. cinerea*, each of which was originally isolated from infected grape berries and maintained on Potato Dextrose Agar (PDA, Sigma). The inoculated leaves were incubated in a growth chamber under a 14:10 hour light:dark period at 25 $\pm$ 3 C. Five days later, the lesion diameter developed around each inoculation point was measured under a stereoscope. In preliminary tests, the lesion size after *Botrytis* infection was 65% less in a high chitinase expressing line as compared to a non-bombarded line.

ii. Greenhouse Assay for Powdery Mildew Resistance

Twenty two lines of Merlot that varied in level of chitinase expression, plus a non-bombarded control were inoculated with *Uncinula necator* in the greenhouse. The plants were sprayed with a suspension of 10^5 spores per ml. A string was tied to the youngest unfolded leaf to standardize the maturity of the leaves for which data was collected. Twelve days after plant inoculation, the number of visible powdery mildew colonies was counted on the leaf with the string and the one immediately below it. The average number of PM colonies per leaf ranged from 1.3 to 17.7 in the transgenics and was 4.1 in the non-bombarded control. To further evaluate mildew growth, the string leaf was harvested from each of 5 replicate plants of the 3 transgenic lines that had the lowest average number of PM colonies per leaf and 3 negative control lines. The leaves were examined microscopically for differences in mildew characteristics. One striking finding was that the number of cleistothecia was significantly lower on the transgenics (200 per half leaf) as compared to the negative controls (750 per half leaf). The 22 transformed lines exhibited a range of PM susceptibility, but the level of tolerance did not correlate with chitinase expression.

iii. Field Evaluation for Powdery Mildew Resistance

Twenty-five lines of Chardonnay and 41 lines of Merlot vines that express the endochitinase gene (*ThEn42*) were planted in the field in New York and California in 1998 and 1999. Vines were rated for the incidence and severity of powdery mildew after natural infection . Six of the Chardonnay lines appear more tolerant to powdery mildew, however, tolerance is not highly correlated with levels of chitinase expression. To date, none of the Merlot lines are significantly tolerant to powdery mildew. Some vine abnormalities were noted, while in the majority of cases the vines appear to be normal.

iv. Botrytis Assay on Fruit Clusters

Mature grapevine clusters from available fruiting transgenic Chardonnay lines were tested for *Botrytis* infection. Clusters were harvested from the field and placed on paper towels and then incubated in a moist chamber with 95 to 97% humidity and

21C for 4 days. This assay enhances expression of *Botrytis* infections that occurred in the field. The clusters were rated for the surface of the cluster infected using the Barratt-Horsfall scale. In these preliminary tests the average rating for the non-transgenic control was 1.0 and the transgenics ranged from 0.2 to 2.0. Disease pressure was very low due to the hot, dry summer weather. We plan to repeat these tests in 2002, using inoculation at the time of flowering.